AD_____

Award Number: W81XWH-11-1-0318

TITLE: De Novo Chromosome Copy Number Variation in Fanconi Anemia-Associated Hematopoietic Defects

PRINCIPAL INVESTIGATOR: Niall George Howlett, Ph.D.

CONTRACTING ORGANIZATION: University of Rhode Island
Kingston, RI 02881

REPORT DATE: April 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2013	Annual	1 April 2012 – 31 March 2013

4. TITLE AND SUBTITLE

De Novo Chromosome Copy Number Variation in Fanconi Anemia-Associated Hematopoietic Defects

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0318

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Niall George Howlett, Ph.D.

E-Mail: nhowlett@mail.uri.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Rhode Island
Kingston, RI 02881

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the second year of the funding period 04/01/2012 until present, we have concluded our efforts to generate pairs of mutant and functionally corrected telomerase-immortalized (hTERT) Fanconi anemia (FA) complementation groups FA-A, FA-D2, and FA-G patient-derived fibroblast lines. We have confirmed re-expression of FANCA, FANCD2, and FANCG in the FA-A + FANCA, FA-D2 + FANCD2, and FA-G + FANCG cells, respectively. We have also confirmed restoration of FANCD2 and FANCI monoubiquitination and functional complementation of the FA-A + FANCA and FA-D2 + FANCD2 cells. In collaboration with the Glover Laboratory in the Department of Human Genetics at the University of Michigan, we have generated a limited number of monoclonal populations of mutant and corrected FA-A, FA-D2, and FA-G lines. Using these monoclonal cell populations, numerous experiments were performed to further isolate clonal populations for de novo copy number variation (CNV) analysis. Despite multiple experiments and the modification of multiple experimental parameters, we were unsuccessful in isolating appreciable numbers of clonal populations for CNV analysis. In collaboration with the Glover Laboratory we have now devised and experimentally optimized an alternative RNA interference-based approach to determine the role of the FA proteins in the suppression of both spontaneous and reactive oxygen species-induced de novo CNVs.

15. SUBJECT TERMS
Fanconi anemia, Copy number variation, Monoclonal cell populations, Functional assays

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	7	19b. TELEPHONE NUMBER *(include area code)*

De Novo Chromosome Copy Number Variation in Fanconi Anemia-Associated Hematopoietic Defects

Grant Number: W81XWH-11-1-0318
Year 2 Progress Report

Table of Contents

Introduction

Fanconi anemia (FA) is a rare disease characterized by developmental defects, progressive bone marrow failure (BMF) and pronounced cancer susceptibility. The FA proteins and the major breast cancer susceptibility gene products BRCA1 and BRCA2 function cooperatively in the FA-BRCA pathway to repair damaged DNA. Recent studies have demonstrated that the FA-BRCA pathway plays an important role in the response of hematopoietic stem and progenitor cells to cellular stresses, and in particular oxidative stress caused by elevated levels of reactive oxygen species (ROS). In our research proposal, we have hypothesized that the FA-BRCA pathway may play an important role in the prevention of genome-wide *de novo* copy number variation. Chromosome copy number variation refers to gains or losses of large (>10 kb) genomic DNA segments. While copy number variation is a feature of normal genetic variation it is also strongly associated with genetic disease, including autism and psychiatric disorders. In addition, several recent studies have demonstrated that hematological malignancies show large numbers of *de novo* somatically acquired copy number variants (CNVs). As with all classes of mutation, an important role for *de novo* CNVs in cancer initiation and progression, as well as BMF, is highly likely. **Importantly, the biological pathways that prevent *de novo* CNV formation, as well as the endogenous and exogenous agents that promote *de novo* CNV formation, remain largely unknown.** We hypothesize that the FA-BRCA pathway, through its role in the suppression and repair of oxidative DNA damage, plays a central role in the prevention of genome-wide *de novo* CNVs. These mutational events are likely to be highly relevant to FA-associated BMF, myelodysplasia (MDS) and progression to acute myeloid leukemia (AML). The major goals of this research proposal are to systematically test the hypothesis that the FA-BRCA pathway plays a major role in the prevention of *de novo* pathogenic CNVs.

Body

With reference to our approved Statement of Work:

Specific Aim1: Determination of the role of the FA-BRCA pathway in the suppression of spontaneous *de novo* CNVs

Task 1. Correction of FA-A, FA-C, and FA-D2 hTERT cells with pLenti6.2/V5-FANCA, -FANCC, and FANCD2, respectively.

Sub-task 1. Selection and expansion of clonal populations

Sub-task 2. Mitomycin C cytotoxicity and clastogenicity assays, assessment of FANCD2 monoubiquitination status *via* immunoblotting and nuclear foci formation – *to determine if the FA-BRCA pathway is functionally restored*

Outcome: As described in our year 1 progress report, over the course of the first year of this funding proposal we focused the majority of our research efforts on the generation of telomerase (hTERT)-immortalized mutant and corrected FA patient-derived fibroblast lines. Specifically, we focused on the following FA complementation groups: FA-A, FA-D2, and FA-G. In our original proposal we indicated that we would use FA-C patient-derived cells, however we chose to focus on FA-G cells instead because of the availability of FA-G lines and the *FANCG* cDNA in our laboratory. To summarize our achievements thus far, we have successfully generated hTERT-immortalized FA-A and FA-A + FANCA cells and, 1) confirmed re-expression of FANCA protein in the FA-A + FANCA cells (Figure 1), 2) demonstrated correction of mitomycin C (MMC)-inducible FANCD2 mono-ubiquitination in the FA-A + FANCA cells (Figure 2), and 3) demonstrated correction of MMC-sensitivity in the FA-A + FANCA cells (results not shown). Thus, the hTERT-immortalized FA-A and FA-A + FANCA cells represent an excellent isogenic system for the study of the role of the FANCA protein in the prevention of *de novo* pathogenic CNVs.

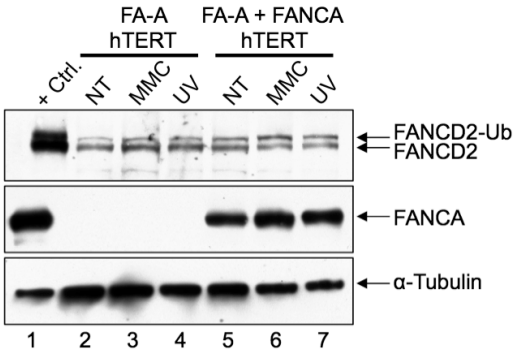

Figure 1. Restored FANCA protein expression and DNA damage-inducible FANCD2 monoubiquitination in FA-A + FANCA hTERT-immortalized cells. NT, no treatment; MMC, 200 nM mitomycin C for 18 h; UV, 6 h post 20 J/m^2 UV-C irradiation.

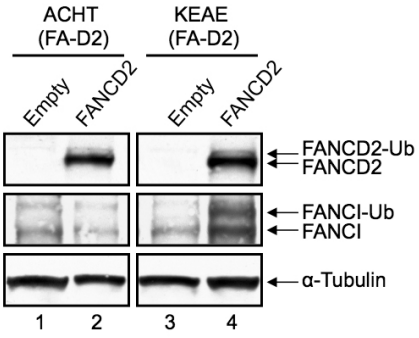

Figure 2. Restoration of FANCD2 protein expression in two independent FA-D2 + FANCD2 hTERT-immortalized lines.

With regard to the FA-D2 complementation group, in year 1 of the funding period, we obtained two mutant and functionally corrected FA-D2 primary lines from Detlev Schlinder of the University of Wuerzburg in Germany. We have hTERT-immortalized these lines and confirmed re-expression of the FANCD2 protein in both the ACHT and KEAE FA-D2 + FANCD2 hTERT lines (Figure 2). Importantly, however, we observed restoration of MMC-inducible FANCI (*FANCD2 paralog*) monoubiquitination in only one of these lines, namely the hTERT-immortalized KEAE (FA-D2) + FANCD2 cells (Figure 2, middle panel, lane 4). Therefore, we chose to use the KEAE FA-D2 cells for further experimentation. Similar to that described for the FA-A complementation group above, during year 2 we have also performed MMC G2/M cell cycle stage accumulation assays with these cells and confirmed functional correction of the KEAE (FA-D2) + FANCD2 cells, consistent with that reported by the Schlinder group. Therefore, similar to the hTERT-immortalized FA-A and FA-A + FANCA cells, the hTERT-immortalized KEAE FA-D2 and FA-D2 + FANCD2 cells represent an excellent isogenic system for the study of the role of the FANCD2 protein in the prevention of *de novo* pathogenic CNVs.

With regard to the FA-G complementation group, we hTERT-immortalized FA-G patient-derived fibroblasts and subsequently infected these cells with the murine moloney leukemia retroviral vectors pMMP-Empty or pMMP-FANCG. While we confirmed re-expression of FANCG in the FA-G + FANCG hTERT cells, we could not detect FANCD2 protein expression or FANCD2 and FANCI monoubiquitination in these cells by immunoblotting (Figure 3), raising some concerns as to whether the FA-G + FANCG cells are fully functionally corrected.

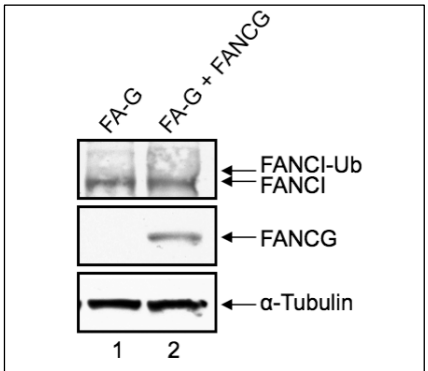

Figure 3. Restored FANCG protein expression in FA-G + FANCG hTERT-immortalized cells.

Task 2 Determination of the frequency of spontaneous *de novo* CNVs in mutant and corrected FA-A, FA-C, and FA-D2 patient cells

Sub-task 1. Selection and expansion of clonal populations of FA-A, FA-A + FANCA, FA-C, FA-C + FANCC, FA-D2 and FA-D2 + FANCD2 cells

Sub-task 2. SNP array analysis of genomic DNA from clonal cell populations – *to be performed at the University of Michigan*

Outcome: Task 2 of specific aim 1 was to determine the frequency of spontaneous *de novo* CNVs in mutant and corrected FA-A, FA-C (*revised to FA-G*), and FA-D2 patient cells. In consultation with our collaborators at the University of Michigan, we decided that it was critical to isolate monoclonal populations of hTERT-immortalized FA-A and FA-A + FANCA, FA-D2 and FA-D2 + FANCD2, and FA-G and FA-G + FANCG cells prior to beginning the CNV analyses experimentation. The reasons for this are that all cells will have a measurable background level of CNVs, and polyclonal cell populations will be mosaic for background CNV levels. For the purposes of our experiments, it is important to isolate genetically homogenous populations, with respect to CNV, prior to initiating our experiments. Therefore, *de novo* CNVs will be clearly distinguishable from pre-existing CNVs in the parental cell population. Thus, in year 2 of the funding period we have sought to isolate monoclonal populations of these cells. This has proven to be an extremely challenging endeavor. Nevertheless, in collaboration with the Glover Laboratory, we have successfully managed to isolate a small number of monoclonal populations of the hTERT-immortalized FA-A and FA-A + FANCA, FA-D2 and FA-D2 + FANCD2, and FA-G and FA-G + FANCG cells. However, it must be emphasized that for the mutant FA-A, FA-D2, and FA-G cells, *yet not the corrected cells*, this was a remarkably inefficient process, discussed in more detail below.

Isolation of monoclonal cell populations: Upon generating these monoclonal cell populations we are now in a position to begin the actual experimentation, i.e. selection and expansion of clonal populations of FA-A, FA-A + FANCA, FA-D2, FA-D2 + FANCD2, FA-G and FA-G + FANCG cells for the analysis of spontaneous *de novo* CNV. Multiple experiments have been conducted in our laboratory towards this goal with limited success. The following are some of the variables that we have modified over the course of our experiments in an effort to isolate appreciable numbers of monoclonal cell populations. We have used several different commercially available tissue culture dishes and flasks, e.g. Corning, Fisher BioLite, and CellTreat. We have tried several different brands, grades and percentages of fetal bovine serum in our tissue culture medium. We have varied the frequency at which we changed our media during the clonal isolation process. We have plated cells at various cell densities. We also purchased a New Brunswick Galaxy 170R tri-gas incubator enabling us to grow cells at physiological oxygen concentrations ($[O_2]_{Phys}$ = 5%), as opposed to atmospheric oxygen concentrations ($[O_2]_{Atmos}$ = 20%). Numerous reports have indicated that primary/hTERT-immortalized cells display significantly improved growth/proliferation when cultured under physiological oxygen concentration, as opposed to atmospheric oxygen concentration (1). We hypothesized that an increased oxidative burden might be contributing to our difficulties in isolating monoclonal cell populations, and that this might be particularly relevant to FA patient-derived cells, as they are well known to be sensitive to reactive oxygen species (2,3). Unfortunately, culturing these cells at $[O_2]_{Phys}$ did not lead to an improved yield of monoclonal cell populations. Thus, despite multiple attempts using multiple variations in experimental conditions, we were unsuccessful in generating appreciable numbers of additional monoclonal populations of the mutant FA cells. We believe that this phenotype may be innately linked to the underlying DNA repair and/or cell proliferation defects of FA cells. Interestingly, the Glover Laboratory has previously attempted to isolate monoclonal populations of hTERT-immortalized Werner syndrome (WS) patient-derived cells without success. WS is characterized by cancer predisposition and accelerated aging and is caused by biallelic mutations in the *WRN/RECQL2* gene (4). Similar to FA, WS patient-derived cells have innate DNA repair defects suggesting that defective DNA repair may negatively impact the clonal expansion of hTERT-immortalized cells in general.

Alternative approach: Because of the considerable difficulties in isolating monoclonal cell populations from our FA-A, FA-D2, and FA-G patient-derived cells, in collaboration with the Glover Laboratory, we have recently devised an alternative approach for assessing the role of the FA proteins in the suppression of spontaneous and ROS-inducible *de novo* CNV. The Glover Laboratory has successfully used an RNA interference approach (RNAi or short-interfering RNA (siRNA)) to interrogate the role of several DNA repair proteins, e.g. RAD51, in the molecular origins of CNV (*Thomas W. Glover, personal communication*). The Glover Laboratory has used two cell types for these experimental approaches: 090 hTERT and HCT116. 090 hTERT are a normal hTERT-immortalized skin fibroblast while HCT116 are a colorectal carcinoma cell line. The Glover Laboratory has recently sent us monoclonal populations of both cells and we are in the process of establishing optimal experimental conditions for the knockdown of FANCA and FANCD2 in these cells. Using these monoclonal HCT116 cells we have optimized conditions for the highly effective knockdown of FANCA and FANCD2 (Figure 4). Thus, we are now poised to conduct experiments to determine the role of FANCA and FANCD2 in the suppression of spontaneous and ROS-inducible CNV using this experimental system. Another advantage of using this system is that these cells have a considerably faster growth rate than the hTERT-immortalized FA patient-derived fibroblast. Therefore, we anticipate that we will be able to generate monoclonal populations of these cells for *de novo* CNV analyses in a relatively short period of time.

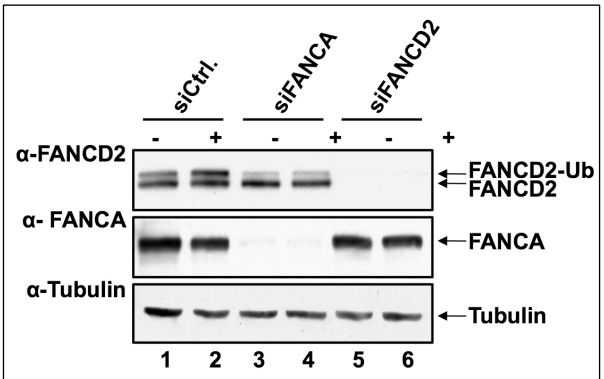

Figure 4. Short-interfering RNA (siRNA) knockdown of FANCA and FANCD2 in HCT116 colorectal carcinoma cells. HCT116 cells were transfected with control non-targeting siRNA (siCtrl.), siFANCA, and siFANCD2 and protein knockdown was assessed by immunoblotting.

Key Research Accomplishments

- We have determined that FA-A + FANCA hTERT cells express robust levels of FANCA protein
- We have determined that DNA damage-inducible FANCD2 monoubiquitination is restored in the FA-A + FANCA hTERT cells
- We have established that the FA-A + FANCA hTERT cells are functionally corrected, i.e. are no longer hypersensitive to the DNA interstrand crosslinking agents MMC
- We have generated FA-G hTERT and FA-G + FANCG hTERT-immortalized cells
- We have determined that the FA-G + FANCG hTERT cells express robust levels of FANCG protein
- We have generated two pairs of FA-D2 hTERT and FA-D2 + FANCD2 hTERT-immortalized cells
- We have determined that the FA-D2 + FANCD2 hTERT cells express robust levels of FANCD2 protein that can undergo monoubiquitination
- We have confirmed DNA damage-inducible FANCI mono-ubiquitination in the KEAE FA-D2 + FANCD2 cells
- We have successfully isolated a small number of monoclonal populations of hTERT-immortalized FA-A, FA-A + FANCA, FA-D2, FA-D2 + FANCD2, FA-G and FA-G + FANCG cells
- We have optimized conditions for depleting FANCD2 and FANCA in monoclonal populations of 090 hTERT and HCT116 cells

Reportable Outcomes

The major reportable outcomes achieved during the first year of funding for this award are the development of multiple cell lines critical for our experimental approach, as described above.

Conclusion

The major conclusion from our studies to date is that the initial goal of isolating monoclonal populations of hTERT-immortalized FA-A, FA-D2, and FA-G patient-derived cells is technically highly challenging and that an alternative approach is required. An alternative RNA interference-based approach has been established and optimized and we are now poised to use this approach to systematically assess the role of FANCA and FANCD2 in the suppression of spontaneous and ROS-inducible CNV and to directly test the hypothesis that the FA-BRCA pathway plays a major role in the prevention of *de novo* pathogenic CNVs.

References

1. Atkuri, K.R., Herzenberg, L.A., Niemi, A.K., Cowan, T. and Herzenberg, L.A. (2007) Importance of culturing primary lymphocytes at physiological oxygen levels. *Proc Natl Acad Sci U S A*, **104**, 4547-4552.
2. Du, W., Adam, Z., Rani, R., Zhang, X. and Pang, Q. (2008) Oxidative stress in Fanconi anemia hematopoiesis and disease progression. *Antioxid Redox Signal*, **10**, 1909-1921.
3. Zhang, X., Sejas, D.P., Qiu, Y., Williams, D.A. and Pang, Q. (2007) Inflammatory ROS promote and cooperate with the Fanconi anemia mutation for hematopoietic senescence. *J Cell Sci*, **120**, 1572-1583.
4. Ozgenc, A. and Loeb, L.A. (2006) Werner Syndrome, aging and cancer. *Genome dynamics*, **1**, 206-217.